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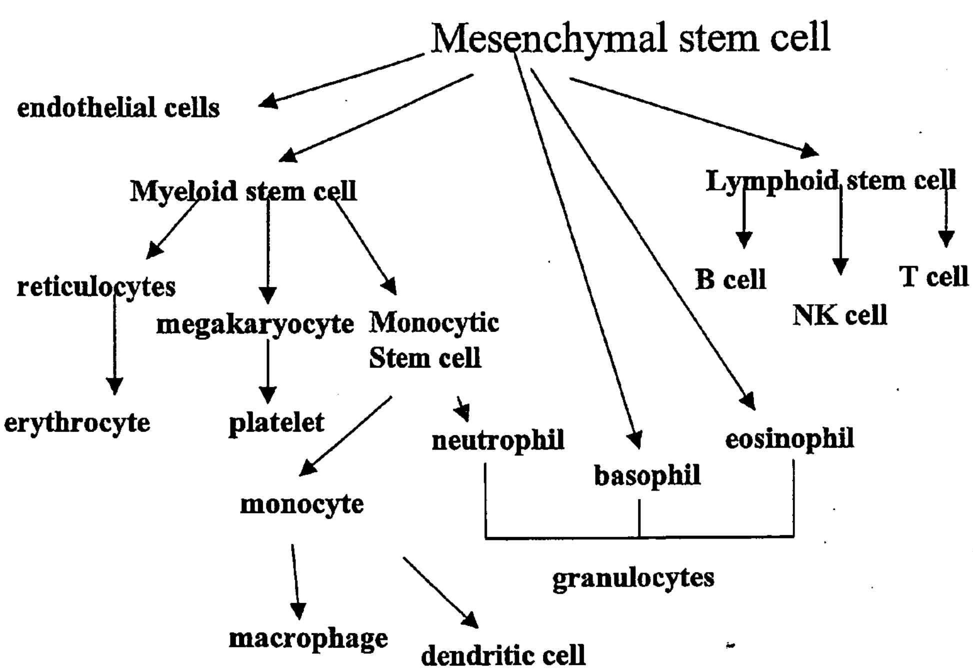
(54) Title: BLOOD PRODUCTS FROM MESENCHYMAL STEM CELLS



(57) Abstract: A method of producing blood products *in vitro* and a method of treatment are provided. The methods include culturing isolated non-SV40 transformed mesenchymal stem cells with growth factors for a time sufficient to produce at least one type of blood products. A method of differentiating mesenchymal cells is also provided. The method of differentiating mesenchymal cells includes culturing isolated non-SV40 transformed mesenchymal stem cells *in vitro* with growth factors and producing at least one blood cell product.

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BLOOD PRODUCTS FROM MESENCHYMAL STEM CELLS

BACKGROUND

1. Technical Field

[0001] The present invention relates to blood products and methods of producing blood products from mesenchymal stem cells.

2. Background Information

[0002] Hematopoietic stem cells (HSCs) and Mesenchymal stem cells (MesSCs) are readily distinguishable cell populations based on cell surface markers. HSCs and MesSCs have previously been thought to give rise to distinct populations of cells and tissues.

[0003] HSCs were known to form blood cells, such as erythrocytes, myelocytes, platelets, dendritic cells, and lymphocytes. HSCs are also known as Bone Marrow stem cells (BMSC) and Marrow stem cells (MSC). HSCs have been derived from bone marrow and are identified as being non-adherent, CD34-positive and CD38-negative cells. Additionally, HSCs have been identified as Lin-negative and HLA DR-positive cells.

[0004] MesSCs form non-hematopoietic tissues such as bone, cartilage, tendon, fat, muscle, and neural cells. MesSCs are oligopotent progenitor cells located in bone marrow, blood, and other tissues that can differentiate into a variety of non-hematopoietic tissues including bone, cartilage, tendon, fat, muscle, and early progenitors of neural cells. MesSCs differentiate into different cell types depending on the interplay of a variety of environmental influences on the cells, including growth factors, and physical environment. MesSCs differ from HSCs in that MesSCs are identified as CD34-negative cells. MesSCs may also be identified as negative for CD45 and positive forCD105, CD59, CD90, CD13, and MHC I after at least one passage in culture.

[0005] Examples of MesSCs differentiation into non-hematopoietic tissues include MesSCs differentiation into mesoderm (fat, cartilage, bone, tendon, cardiac muscle and skeletal muscle; Pittinger, MF et al., 1999, *Science*, 248: 385-389; Jaiswal, N et al., 1997, *J. Cell Biochem.,* 64(2):295-312; Shakibaei, M et al., 1997, *Cell Biol. Int.,* 21(2):115-25.; Fukuda, K, 2001, *Artif. Organs.* 25(3):187-93.) and into ectoderm (neuronal cells, Deng et al. 2001, *Biochem. Biophys. Res. Commun.,* 282(1):148-52.; Woodbury et al., 2000, *J. Neurosci. Res.*, 61(4):364-70.). After transplantation of MesSCs into infarcted myocardium of rats, transplanted cells differentiated into cardiac muscle and were detected weeks later in regenerating heart tissue, as detected by a marker gene (Toma, C et al., 2002, *Circulation*, 105(1):93-8.). In other animal models, MesSCs were shown to differentiate into brain and spinal cord in injured or knockout animals, leading to improvement or healing of injured neural tissues. (Hofstetter et al., 1999, *Proc. Natl. Acad. Sci. U.S.A.,* 99(4):2199-204.).

[0006] Some studies have suggested that MesSCs may be beneficial to support hematopoietic cells, such as in bone marrow transplantation, immunoregulation, and graft facilitation. However, MesSCs have not been cultured *in vitro* to form blood cells.

[0007] Advantages for using MesSCs to form blood products include the ability to greatly expand the MesSCs in culture after isolation. Unlike HSCs that do not propagate well in culture, MesSCs may be greatly expanded in culture and still retain the ability to differentiate into a plurality of blood products. For example, a small amount of bone marrow aspirate may provide enough MesSCs to differentiate into large numbers of cells to form the blood products of the present invention.

[0008] Thus, methods for providing blood products from MesSCs are desirable.

BRIEF SUMMARY

[0009] Accordingly, one object of the invention is to provide a method of producing blood products *in vitro* comprising culturing isolated, non-SV40 transformed, mesenchymal stem cells with growth factors for a time sufficient to produce at least one type of blood products.

[0010] Another object of the invention is to provide a method of differentiating non-SV40 transformed mesenchymal stem cells including culturing isolated mesenchymal stem cells *in vitro* with growth factors and producing at least one type of blood cell products.

[0011] Another object of the invention is to provide a method of treatment of a patient in need of a blood product, said method comprising delivering a therapeutic amount of at least one type of blood products produced by culturing isolated, non-SV40 transformed, mesenchymal stem cells *in vitro* with growth factors for a time sufficient to produce at least one blood product.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1 is a diagram of mesenchymal stem cell differentiation to form various blood products of the present invention;

[0013] FIG. 2 is a graph showing growth and doubling characteristics of the isolated mesenchymal stem cells of the present invention;

[0014] FIG. 3 is a graph showing blood cell counts after transplantation in mice with the MesSCs of the present invention; and

[0015] FIG. 4 is diagram showing the subpopulation of blood cells in transplanted mice.

DETAILED DESCRIPTION OF THE DRAWINGS AND THE PRESENTLY PREFERRED EMBODIMENTS

[0016] The present invention utilizes isolated, cultured mesenchymal stem cells (MesSCs) to produce blood products in the form of cells. Blood products produced with this method include those conventionally formed from hematopoietic stem cells. The terms "blood products" or "type of blood products" as used herein refers to all cells found in peripheral blood, including stem cells, immature, and mature cell populations. By way of example, the blood products include, but are not limited to, hematopoietic stem cells formed from cultured MesSCs, myeloid stem cells, endothelial cells, lymphoid stem cells, antigen presenting cells, erythroid cells, and megakaryocytes, and populations of cells derived therefrom. FIG. 1 diagrams the main developmental stages in blood cell formation. The process of blood cell formation is a continuum of proliferation and differentiation from the stem cells to the mature cells found in circulating blood. FIG. 1 illustrates exemplary blood products that may be formed from the cultured MesSCs. For example erythrocytes, macrophages and dendritic cells may all be derived from myeloid stem cells. However, the blood products illustrated in FIG. 1 are not meant to be limiting.

Isolation of MesSCs

MesSCs in accordance with the present invention can be identified by cell surface expression markers. Suitable MesSCs have a majority of the surface markers expressed as shown in Table I.

Table 1: **Expression of surface markers on MesSCs**

Marker	Expression
CD11a (LFA-1)	-
CD11b	-
CD13	+
CD31 (PECAM)	-
CD34	-
CD40	-
CD40L	-
CD45	-
CD49d (VLA-4)	+
CD58 (LFA-3)	+
CD59	++
CD80 (B7.1)	-
CD86 (B7.2)	-
CD90 (Thy 1)	+
CD105 (Endoglin)	+
CD117 (c-kit)	-
CD133 (AC133)	-
CXCR4	-
CCR7	-
CLA	-
ABCG2	-
KDR (flk-1, VEGFR-2)	+/- (low)
SDF-1	+/- intracellular
MHC I	+
MHC II	-

[0017] In an embodiment of the present invention, described herein, the MesSCs used to produce blood products may be derived from bone marrow or blood, preferably bone marrow aspirates. However, the MesSCs may be derived from any source of cells known to one of skill in the art, including, but

not limited to bone marrow, blood, dermis, and periostium. The term culturing as used herein includes growing MesSCs, expanding MesSCs, and differentiating MesSCs to form blood products. The term SV40 transformed as used herein refers to the transformation of cells *in vitro* into a cell line which is able to grow with unrestricted doublings, having an infinite life span using an SV-40 large T antigen to transform cells having a limited number of population doublings into a cell line, having infinite population doublings. Non-SV40 transformed cells have not been transformed with the SV-40 large T antigen. The term stem cell as used herein refers to a immature cell which is capable of giving rise to other cell types.

Culturing, Expanding and Differentiating MesSCs

[0018] In a preferred embodiment of the present invention, the MesSCs are isolated from bone marrow aspirates using a Percoll® gradient, as described below. The growth characteristics of the isolated MesSCs are shown in FIG. 2.

[0019] After Percoll® gradient isolation, the MesSCs may be cultured and expanded *in vitro*. Following passage in culture, the MesSCs may be grown in the presence of differentiation media and growth factors to produce blood products. By way of example, the media may include, but is not limited to, the following growth factors added individually or in combinations thereof: stem cell factor (SCF), thrombopoietin (TPO), flt-3 ligand (FL), interleukins, including interleukin-3 (IL-3) and interleukin-6 (IL-6), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), erythropoietin (Epo), vascular-endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), epidermal growth factor (EGF), leukaemia inhibitory factor (LIF), and hydrocortisone (HC). Alternatively, MesSCs may be grown in the presence of differentiation media and growth factors without passaging the MesSCs prior to differentiation. The blood cell products formed *in vitro* from the MesSCs will depend on the culture conditions selected for differentiation and are described in more detail below.

[0020] The cells isolated from the bone marrow may be used immediately, or alternatively, the cells may be stored for later expansion and/or differentiation. For example, freshly isolated bone marrow cells may be stored, preferably cryopreserved, as is routinely done in the field of bone marrow transplantation. The freshly isolated bone marrow comprises a mixture of cells, including MesSCs and HSC. The stored bone marrow cells may separated on a Percoll® gradient, isolated, cultured, and differentiated at a later time without substantial loss of proliferative or differentiation capacity. Isolated MesSCs may also be stored, preferably cryopreserved, after Percoll® centrifugation, and prior to *in vitro* culture. Cultured MesSCs may also be stored, preferably cryopreserved, in early passage after plating onto plastic in cell culture, such as P0 or P1 without substantial loss of proliferative or differentiation capacity. Potentially all blood products, at any step in the process from harvested bone marrow to differentiated blood products, may be stored for a period of time, prior to delivery to a patient. Storage of the blood products may be any type of storage known to one of skill in the art. Storage of blood products may be advantageous for many reasons, including, but not limited to, repeated administrations given to a patient, autologous donation for later use, characterization of cell populations and later use, and others.

[0021] In a preferred embodiment, the MesSCs are isolated from bone marrow aspirates. The bone marrow is obtained from autologous or allogeneic donors according to procedures known to one of skill in the art. Once the bone marrow is obtained from the donor, the MesSCs are isolated from the bone marrow aspirate using a Percoll® gradient.

[0022] In a preferred embodiment, a discontinuous gradient is prepared. Preparation of the gradient and separation of the MesSCs have been described in German Patent Application Serial No. 103 36 152.9, filed August 6, 2003, and in International Application No. PCT/EP2004/008865, filed August 6, 2004 (both applications being entitled "Purification procedure for mesenchymal stem cells"), both documents of which are incorporated by reference herein in their entirety.

[0023] In a preferred embodiment, Percoll® solution with the density 1.124 g/ml (Fa. Biochrom, Berlin, Germany) is used to prepare a discontinuous density gradient. Dilutions of the basic solution are prepared with PBS (Phosphate-buffered salt solution without Ca^{++} and Mg^{++}, Gibco, Karlsruhe, Germany) according to the formula:

$$V[\%] = \frac{(D'-D\%) \times 10^2}{D''-D\%}$$

where:

D′ desired final density (g/ml)
D″ high basic density (g/ml)
D% density of the iso-osmolale diluent (g/ml)
V% volume-percent of the basic solution with high density.

Separation-solutions using this method can be prepared using a variety of densities, preferably with the following densities: 1.050, 1.063, 1.068, 1.077, 1.088 and 1.100 g/ml.

[0024] The bone marrow aspirates are diluted 1:1 with PBS and layered onto the Percoll® gradients and centrifuged for 20 min with 800 x g at room temperature (RT). The low-density cells are isolated from higher density red blood cells and mononuclear hematopoietic cells as describe below in Example 1. The low-density cells are selected for high proliferative potential, based on characterisation in further growth, phenotypic and differentiation assays. Alternatively, the MesSCs may be isolated by any method known to one of skill in the art.

[0025] The low-density cells comprising MesSCs are collected, washed twice in PBS, resuspended in growth medium comprising DMEM/low glucose (Gibco) supplemented with 10% preselected fetal calf serum (FCS) (BioWhittaker, Apen, Germany) and 1% penicillin/streptomycin (Gibco). The FCS was preselected by comparing growth characteristics for MesSCs grown in DMEM/LG + 10% FCS. The MesSCs were tested in passage P4 for the

MesSCs' phenotype in FACS analysis and differentiation assays, including adipo-, osteo- and chrondrogeneic potential. The FCS was selected for promoting differentiation and for the greatest expansion of the test MesSCs. The isolated cells comprising MesSCs are counted in a Neubauer chamber. For initial seeding, 1 x 10^7 cells are seeded in tissue culture flasks with 25 cm^2 growth surface and grown in an incubator with 5% CO_2 and ≥ 97% humidity to confluency of about 90%. The isolated cells comprising MesSCs are preferably CD34-negative, plastic adherent, fibroblast-like cells. The isolated MesSCs grow through 4 to 10 passages, depending on the quality of the donor material, with an approximately constant doubling rate as shown in FIG. 2. Eventually, the proliferation potential decreases and the MesSCs cease growing. During the active proliferation phase, the isolated MesSCs display cell surface markers specific for MesSCs, including CD 105, CD 59, CD 90, CD 13 and MHC I. The MesSCs are negative for hematopoietic markers, including CD 34 and CD 45. For MesSCs differentiation into the plurality of blood products, various differentiation media and growth factors may be used. For example, a population of leukocytes expressing the pan leukocyte marker, CD 45, may be obtained within approximately two weeks of culture. The isolated MesSCs, which are CD34- and CD45-negative and at least CD90- and CD105-positive, may be grown in a differentiation media to produce a population of blood products comprising leukocytes, expressing CD45. The differentiation media for obtaining the CD45 positive cells includes IMDM as basal medium, supplemented with 10% FCS and 10% horse serum (HS), 1 x 10^{-6} M HC mixed 1 : 1 with methylcellulose No. 4535 (CellSystems Biotech, containing the proliferation increasing cytokines SCF, IL-3, IL-6, G-CSF, GM-CSF. Epo is also added to the culture. Blood products comprising CD45 positive cells are obtained within 2 weeks of incubation. The medium for the cultured MesSCs is changed every second day.

[0026] To obtain blood products comprising myeloid stem cells, the MesSCs are first cultured in StemSpan (CellSystems Biotech) as serum free basal medium supplemented with SCF, TPO, GM-CSF and FL for two weeks to prestimulate the MesSCs. The medium for the cultured MesSCs is

changed every second day. Thereafter, the MesSCs are harvested, resuspended in serum free methylcellulose No. 4436 (CellSystems Biotech, containing the proliferation increasing cytokines SCF, IL-3, IL-6, G-CSF, GM-CSF, and Epo) and incubated for an additional 2 weeks. The blood products formed may include monocytes/macrophages, indicated by CD 14 positive staining and granulocytes, including neutrophils, eosinophils and basophils, indicated by CD 16 positive staining. Additionally, BFU-E (burst forming unit-erythroid) structures may be formed from the myeloid culture conditions and identified by morphology and immunohistochemical staining with an antibody to glycophorine A.

[0027] For blood products comprising endothelial cells, the MesSCs were seeded in chamber-slides in StemSpan (StemCell Tech) as serum free basal medium supplemented with VEGF, bFGF and HGF and 10^{-6} M HC. The MesSCs were incubated for 3 weeks with medium changes every second day. Alternatively, MesSCs were prestimulated for two weeks in StemSpan supplemented with SCF, TPO, FL followed by differentiation for two weeks in StemSpan supplemented with VEGF, bFGF and IGF. The blood products comprising endothelial cells are identified by immunohistochemical staining for CD 31, CD 34, vWF (von Willebrand Factor), and KDR (also known as VEGFR2, Vascular Endothelial Growth Factor Receptor 2).

Confirming Cell Types

[0028] After differentiation, the blood products formed may be optionally cytochemically stained to confirm the cell type(s) present in the culture. Preferably, the blood product cell type will be confirmed using immunocytochemistry, more preferably, the cell type will be confirmed using cell surface marker expression of Cluster of Differentiation (CD) epitopes.

[0029] Within each lineage and between each lineage of blood product formed, antigens, such as CD antigens, are expressed differentially on the surface and in the cytoplasm of the cells in a given lineage. The expression of one or more antigens and/or the intensity of expression can be used to distinguish between maturational stages within a lineage and between

lineages. The blood products formed from the differentiated cells may then be administered to a patient.

[0030] As described above, the blood products derived from MesSCs and the MesSCs are stained for cell surface marker expression to identify the blood products formed. In an embodiment of the present invention, the blood products are harvested, washed in PBS and centrifuged on slides for cytospin preparations. Alternatively, adherent blood products, such as endothelial cells, may be grown directly on chamber-slides for subsequent staining. The slides are air-dried, fixed for 10 minutes in ice-cold acetone. The acetone-fixed cells may be stored at -20°C until staining.

[0031] For staining, slides are warmed to RT and blocked with 5% normal goat serum in PBS for 10 min. After removal of the blocking solution, the cytospin preparations or chamber-slide preparations are incubated with titrated amounts of primary antibodies for 30 min at RT in a humidified chamber. The staining is followed by 2 washes in PBS for 10 min. each at RT and incubation with titrated amounts of fluorochrome-labeled secondary antibodies for 30 min. in a humidified chamber. Slides are washed twice with PBS and stained for 1 min. with DAPI to visualize the cell nucleus, followed by 2 washes with PBS for 10 min. A short wash of the slides with aqua dest. is followed by fixation for 5 min. in absolute ethanol and then air-drying. The slides are covered with fluoromount (Southern Biotech, Eching, Germany) and glass cover slipped and stored until analysis at 4°C. Alternative methods for staining are known to the skilled artisan or will become apparent from the instant description of the invention.

[0032] The analysis of the stained blood products is carried out using a high-power light and fluorescence microscope. As described above, the following staining, using the identified markers alone or in combinations thereof, has been observed for the identified blood products:

a) myeloid stem cells positive for CD34, CD133, CD14, CD16, CD45;
b) erythroid cells positive for Glycophorin A;
c) megakaryocytic cells positive for CD41;

d) endothelial cells positive for vWF, CD31, CD34, KDR;
e) lymphoid cells positive for CD3 and
   i) T cells positive for CD4, CD8;
   ii) NK cells positive for CD56;
   iii) NK-T cells positive for CD3, NK1.1;
   iv) B cells positive for CD10; CD19; CD20;
f) dendritic cells positive for CD80, CD86, MHC I, MHC II, CD1a, CD40.

Delivery of blood products

[0033] Delivery of the differentiated mesenchymal stem cells may be by injection, infusion or instillation of the blood products into the patient. The blood products may be injected, infused, or instilled directly into a desired target organ or alternatively the blood products may be injected intravenously, intra-arterially, or intraperitoneally. Any delivery method for cells, commonly known in the art, may be used for delivery of the blood products formed from MesSCs.

[0034] The blood products formed from the cultured MesSCs may be used to treat patients in need of a blood product. In certain embodiments, a therapeutically effective dose of blood products is delivered to the patient. An effective dose for treatment will be determined by the body weight of the patient receiving treatment. A therapeutic dose may be one or more administrations of the therapy. For example, where recovery of the blood cell numbers has been reached within about 2 weeks, one infusion of MesSCs may be sufficient for treatment. However, numerous applications of MesSCs blood products are not detrimental to the patients and may provide advantages over a single administration. An exemplary dose of a blood product may be about 1-50 x 10^6 per kilogram body weight.

[0035] The blood products used for treatment may be derived from autologous donor MesSCs or allogeneic donor MesSCs. Use of autologous MesSCs eliminates concerns regarding immune reactions provoked by the allogeneic cells. Additionally, repetitive administrations of autologous MesSCs are possible. Allogeneic MesSCs will be provided to the patient using matching criteria for organ transplantation commonly known to one of skill in the art. Allogeneic MesSCs derived from a compatible donor may also

be advantageous for producing blood products for administration to a patient. Allogeneic blood products may be administered, for example, when the bone marrow in a patient who is in need of blood products may be a poor source of adequate numbers of usable stem cells because the patient may have received bone marrow toxic drugs or radiation or may have leukemia or bone marrow metastasis, when a patient may refuse or may not be able to consent to the harvesting of his/her own bone marrow cells, and when the bone marrow-derived stem cells from a compatible living-related or unrelated donor may be of superior quality and quantity compared to the recipient's own stem cells.

[0036] Examples of patients that may receive autologous blood products include cancer/leukemia patients in need of a bone marrow transplant and no compatible donor is available, cancer/leukemia patients undergoing chemotherapy, and patients developing thrombocytopenia after bone marrow transplant or chemotherapy. Additionally, patients who, after bone marrow transplantation or chemotherapy, have low erythrocyte or granulocyte counts may receive blood products derived from autologous MesSCs. Autologous blood product treatment may be advantageous for patients with allergic and autoimmune reactions.

[0037] Examples of patients that may receive allogeneic blood products include the same patients exampled for autologous blood product recipients listed above. Allogeneic cell preparations of MesSCs may be advantageous in that populations of specific cell types may be generated, screened, and stored. Thus providing a pool of specialized blood products including platelets, erythrocytes, leukocytes or specialized cells thereof, lymphocytes, and dendritic cells that are screened in advance of administration to the patient and certified to be, for example, disease free.

[0038] A further object of the invention is the use of at least one type of blood products obtainable by culturing isolated non-SV40 transformed mesenchymal stem cells *in vitro* with growth factors for a time sufficient to produce at least one type of blood products for preparing a pharmaceutical composition for treating of patients suffering from leukemia,

thrombocytopenia, leukopenia, granulocytopenia, lymphocytopenia (such as HIV patients), aplastic anemia, and/or autoimmune disease with or without bone marrow involvement, patients after chemotherapy (including high-dose chemotherapy), total body irradiation or irradiation of single parts of the body (including irradiation of bones and organs) as well as patients with vascular, ischemic (including cardiac ischemia), and/or malignant disease. In particular, a further object of the invention is the use of at least one type of blood products obtainable by culturing isolated non-SV40 transformed mesenchymal stem cells *in vitro* with growth factors for a time sufficient to produce at least one type blood products for preparing a pharmaceutical composition for treating of patients suffering from anemia due to acute leukemia, due to chronic leukemia, due to osteomyelofibrosis, due to aplastic anemia, due to thalassaemia, due to sickle cell disease, due to loss of blood, e.g. after an accident, due to chemotherapy, due to medical drugs other than chemotherapy, due to radiation, and/or due to abuse of toxic compounds, and for treating of patients suffering from leukopenia due to acute leukemia, due to chronic leukemia, due to osteomyelofibrosis, due to aplastic anemia, due to thalassaemia, due to sickle cell disease, due to loss of blood, e.g. after an accident, due to chemotherapy, due to medical drugs other than chemotherapy, due to radiation, and/or due to abuse of toxic compounds, and for treating of patients suffering from thrombocytopenia due to acute leukemia, due to chronic leukemia, due to osteomyelofibrosis, due to aplastic anemia, due to thalassaemia, due to sickle cell disease, due to loss of blood, e.g. after an accident, due to chemotherapy, due to medical drugs other than chemotherapy, due to radiation, and/or due to abuse of toxic compounds, and for treating of patients suffering from vascular diseases, such as autoimmune vasculitis, arterial occlusive disorders, venous occlusive disease, and/or artherosclerosis, and for treating of patients suffering from ischemic diseases, such as coronary heart disease, stroke, acute renal failure, and/or claudicatio intermittens.

[0039] A further object of the invention is the use of at least one type of blood products obtainable by culturing isolated non-SV40 transformed mesenchymal stem cells *in vitro* with growth factors for a time sufficient to produce at least one type of blood products for preparing a pharmaceutical composition for diagnosis of cancers and metastasis, wherein said at least one type of blood products is labelled with radioactive compounds. When diagnosing cancers and metastasis, the type of blood products (or cells, respectively) are infused and detected in the patient's body by means and methods well known to the skilled artisan.

[0040] Preferably, in connection with the above-mentioned use, said at least one blood product comprises cells selected from the group consisting of myeloid stem cells, endothelial cells, lymphoid stem cells, dendritic cells, erythroid cells, and megakaryocytes.

[0041] Further, the invention relates to blood products obtained by the methods of the invention, ie. the method of producing blood products *in vitro* or the method of differentiating non-SV40 transformed mesenchymal cells of the invention, respectively. As already outlined above, said blood products comprise cells selected from the group consisting of myeloid stem cells, endothelial cells, lymphoid stem cells, dendritic cells, erythroid cells, and megakaryocytes.

[0042] The invention is further described and exemplified by the following, non-limiting examples.

Examples:

[0043] Example 1 Isolation of MesSCs

[0044] 5 ml of each density 1.050, 1.063, 1.068, 1.077, 1.088 and 1.100 g/ml. of Percoll® (described above) were carefully layered onto each other into a 50 ml-tube. 10 ml of bone marrow were diluted with 10 ml PBS and carefully layered onto the density gradient. In parallel, as controls a) 1 ml bone marrow diluted with 1 ml PBS was layered onto 3 ml Ficoll with a density of 1.077 g/ml

in a 15 ml-tube (designated as stem cells SC; conventional method to separate mononuclear cells as well as MesSCs) and b) each separate 1 ml bone marrow diluted with 1 ml PBS was layered onto 3 ml Percoll with a density of 1.068 g/ml (designated as LD = low density) or 1.077 g/ml (designated as MNC = mononuclear cells) in a separate 15 ml-tube.

[0045] All tubes were centrifuged for 20 min at room temperature with 800g without break. The plasma mixed with PBS was removed from the tubes and each fraction was collected into a separate tube. After washing the cells twice with PBS for 10 min with 400g, the erythrocytes contained in fractions F4-F6 (F1 = lowest density 1.050 g/ml, F6 = highest density 1,100 g/ml) were lysed with hemolysis buffer, the cells washed again in PBS, resuspended in cultivation-medium DMEM/LG (Dulbeccos's Modified Eagle Medium/low glucose, Gibco) + 1% penicillin/streptomycin + 10% preselected fetal calf serum and counted with trypan-blue in a Neubauer chamber. For a growth area of 25 cm^2 1 x 10^7 cells were seeded in 5 ml medium. After 3 days the nonadherent cells were washed off with PBS. Cells were fed every 3 days with fresh medium and incubated until reaching confluence of about 80-90% assessed by phase contrast microscopy. At this time point the culture was designated as P0.

[0046] For demonstrating the CFU-F (colony forming unit-fibroblast; as proof for proliferating activity of separate fractions of MesSCs) 10^6 cells of each fraction as well as LD and MNC control cells were seeded in a well of 6-well plates in 3 ml medium. The cells were incubated in an incubator with 37°C and 5% CO_2. After 3 days the nonadherent cells were washed off with PBS. Cells were fed every 3 days with fresh medium. The CFU-F were washed with PBS after an incubation period of 14 days and stained with 1% crystal violet.

[0047] For passaging the cells, the medium was removed, the plates washed once with PBS, incubated for 5 min with 0.25% trypsin-EDTA and the cells resuspended in medium and counted in a Neubauer chamber. 500 cells/cm^2 were seeded into a new tissue culture flask and designated as P1.

[0048] Example 2 Isolation and Differentiation of MesSCs

[0049] Aspirated bone marrow was diluted 1:1 with PBS (Gibco, Karlsruhe, Germany), layered onto Percoll®-solution (Biochrom, Berlin, Germany) with density 1.068 g/ml and centrifuged for 20 min with 800g at RT. Low-density cells were collected, washed twice in PBS, resuspended in growth medium DMEM/low glucose (Gibco) supplemented with 10% preselected FCS (BioWhittaker, Apen, Germany) and 1% penicillin/streptomycin (Gibco) and counted in a Neubauer chamber. For initial seeding, 1 x 10^7 cells were put in tissue culture flasks with 25 cm^2 growth surface and grown in an incubator with 5% CO_2 and ≥ 97% humidity to confluency of ca. 90%. Passaging was carried out by removing the culture medium, washing the growth surface with PBS and incubation with trypsin/EDTA (Gibco) for 5 min. The cells were resuspended in growth medium, counted and 500 cells/cm^2 seeded into new culture flasks.

[0050] For hematopoietic differentiation *in vitro* cultured MesSCs from the 2^{nd} – 4^{th} passage were used.

[0051] For differentiation into leukocytes, 1 x 10^5 cells were seeded on an area of 0.8 cm^2 in chamber slides and fed 3 times a week with differentiation medium. This medium contains a basic medium IMDM (Gibco) plus 10 % FCS plus 10 % HS (horse serum, both CellSystems Biotech, St. Katharinen, Germany) and 10^{-6} M hydrocortisone (Sigma, Deisenhofen, Germany) and is mixed 1 : 1 with methylcellulose No. 4535 (CellSystems Biotech, containing the proliferation increasing cytokines SCF, IL-3, IL-6, G-CSF, GM-CSF) and Epo.

[0052] Formation of round cells was noted as early as day 5 in several of these cultures, at day 10 colonies of about 100 cells are produced. Colonies were picked and cytospun, the chamber slides were washed once with PBS, all slides air-dried, fixed for 10 min in ice-cold acetone and stored at – 20°C until staining. Alternatively, cells cultured in chamber slides may be typsinized for FACS analysis. The trypsinized cells were incubated with an anti-CD34 antibody or the respective isotype-matched immunoglobulin and analyzed on a Becton Dickinson FACScan.

[0053] For staining, slides were adapted to room temperature (RT) for some minutes and blocked with 5% normal goat serum in PBS for 10 min. After removal of the blocking solution the cytospins were incubated with titrated amounts of primary antibodies for 30 min at RT in a humidified chamber. For detection of myeloid, megakaryocytic and erythroid cells antibodies directed against CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany), CD34, CD14, CD16, CD45, CD41 and Glycophorin A (all antibodies: Dako) were included. For each antibody the appropriate isotype control was carried out identically to the staining for antibodies.

[0054] The staining was followed by 2 washes in PBS for 10 min each at RT and incubation with titrated amounts of the fluorochrome-labeled secondary antibody Alexa 594 (Molecular Probes, Göttingen, Germany) for 30 min in a humidified chamber. Slides were washed 2x with PBS and stained for 1 min with DAPI (Sigma) to visualize the nucleus, followed by 2 washes with PBS for 10 min. A short wash with aqua dest. was followed by fixation for 5 min in absolute ethanol and air-drying. The slides were covered with fluoromount and glass cover slips and stored until analysis at 4°C.

[0055] The analysis was carried out at a high power light and fluorescence microscope. Using this approach, CD45-, CD133- and CD34-positive cells were detected in the clusters described above.

[0056] Example 3 Differentiation of MesSCs into Myeloid and Erythroid Cells

[0057] To obtain differentiation of MesSCs into myeloid and erythroid cells, 1 x 10^5 cells from the 2^{nd} – 4^{th} passage were seeded on a culture area of 2 cm^2 and pre-stimulated for 2 weeks with serum free differentiation medium consisting of the basal medium StemSpan (CellSystems) supplemented with the cytokines SCF (100 ng/ml), TPO (10 ng/ml), GM-CSF (10 ng/ml) and FL (50 ng/ml)(all from Tebu, Offenbach, Germany). Cells were fed 3 times a week.

[0058] Day 14 cells are taken out by heavy pipetting or trypsinization and cultivated in serum-free methylcellulose (CellSystems No. 4435, containing the proliferation increasing cytokines SCF, IL-3, IL-6, G-CSF, GM-CSF and

erythropoietin). Following 14 days of cultivation single colonies and differentiated cells were taken out and examined after cytospin by morphology and immunochemistry with antibodies against the surface antigens of myeloid and erythroid characteristics as described in example 2.

[0059] Using this approach, CD34, CD133, CD14, CD16, CD45, CD41 and Glycophorin A positive cells were detected.

[0060] Example 4 Differentiation of MesSCs into Endothelial Cells

[0061] To obtain differentiation of MesSCs into endothelial cells, 1×10^5 cells from the 2nd – 4th passage were seeded on a culture area of 0.8 cm^2 and stimulated for 3 weeks with serum free differentiation medium including the basal medium StemSpan (CellSystems) supplemented with the cytokines SCF (100 ng/ml), VEGF (50 ng/ml), bFGF (10 ng/ml), HGF (50 ng/ml) (all from Tebu) and 10^{-6} M HC. Alternatively, MesSCs were prestimulated for two weeks in serum free medium supplemented with SCF (100 ng/ml), TPO (10 ng/ml) and FL (50 ng/ml), followed by differentiation in medium plus VEGF (50 ng/ml), bFGF (10 ng/ml), and IGF (10 ng/ml) for two weeks. Cells were fed 3 times a week. After finishing the differentiation slides were air-dried, fixed for 10 min in ice-cold acetone and stored at –20°C until staining.

[0062] Cells were stained according to the procedure described above with antibodies directed against vWF, CD31, CD34 and KDR. Positive cell staining was detected for CD34 and vWF or CD31 and KDR.

[0063] Example 5 Transplantation of MesSCs

[0064] MesSCs were transplanted into stem cell ablated mice to demonstrate the pluripotent potential of the MesSCs of the present invention.

[0065] MesSCs for transplantation were generated from adult male C57Bl/6J mice of the Ly 5.2 phenotype. Cells from femurs and tibiae were seeded in DMEM/Ham´s F12 medium (generally regarded as medium insufficient supporting growth of hematopoietic stem cells) supplemented with 20% FCS, 1% penicillin/streptomycin and1% glutamine. The FCS was selected for rapid growth of plastic adherent growing cells and for lack

of support for growth of hematopoietic cells. Non-adherent cells were removed after 3 days and cultures fed twice a week until confluence. During early passages, up to P5, cells were seeded with 1×10^5 cells/ cm^2. Later passages were seeded with 1000 cells/ cm^2. Cells were passaged in culture up to 9 times over a period of about 6 months. Cells at P8 were determined to be negative for CD45 and CD34, positive for Sca-1, CD90 and CD59 and low positive for CD117 (c-kit).

[0066] For MesSC transplantation, recipient C57Bl/6J mice of the Ly 5.1 phenotype were lethally irradiated with a 9.5 Gy dose of gamma irradiation and transplanted with 1×10^6 MesSCs cultured as described above. Blood cell counts and interim analyses of reconstitution with donor cells were performed from peripheral blood taken retroorbitally. The blood cell counts are shown in FIG. 3 and were performed using a Coulter® blood cell counter. Analysis of reconstitution was performed using cells labelled with fluorochrome-conjugated antibodies against a donor-antigen CD45.2 (Ly 5.2) and analyzed on a FACScan from Becton Dickinson to distinguish donor and recipient cells. The results of the reconstitution analysis are listed below in Table 2 where the percent reflects the percent of total cells that were of donor origin.

Table 2: Recipient Reconstitution

Weeks	% Donor origin (+/- S.D.)
8	9.3 ± 5.3 % (0.1-19.3 %)
15	6.9 ± 2.6 % (3.4-13.7 %)
24	15.6 ± 6 % (10-24.5 %)

[0067] Results show that mice can be reconstituted with MesSCs of the present invention leading to normal white blood cell counts after about 6 weeks and out of the danger zone for the acute risk of infection after about 3 weeks.

[0068] Analysis of cell composition of the individual tissues from MesSC-transplanted mice is shown for bone marrow, thymus and

peripheral blood in Tables 3, 4 and 5, respectively. In general, the transplanted mice showed a low donor chimerism in bone marrow and thymus and up to 35% in peripheral blood (FIG. 4). Population analysis reveals that the donor cells mainly reconstituted the myeloid compartment and in part the lymphoid cells.

Table 3: Bone Marrow Analysis

	% donor type (dt)	% dt+ CD45+	% dt+ c-kit+	% dt+ B220+	% dt+ Ter119+	% dt+ CD3+
Mouse 2	2.3	Nd	6	7.8	4.6	17.3
Mouse 3	2.7	2.4	1.8	8.1	5.5	13.5
Mouse 5	4.5	5	11.2	17.1	7.6	20.6
Mouse 6	2.3	2.2	5.8	3.5	5.4	6.7
Mouse 7	2.8	3.1	7.2	3.4	4.8	12.3
Mouse 8	4	3.5	4.9	3	7.6	14.2
Mouse 9	3.5	4	7.7	6.1	5.3	10.3
Mouse 10	3.1	3.6	6.8	4.7	5.3	14.9
Mouse 11	2	2	5	2.9	5	11.9
Mouse 12	3.6	6.2	28.2	27.5	4.4	23.2
Mouse 14	2.2	2.2	5	4.3	2.9	8.1
Mouse 18	2.9	2.7	4.8	2.5	2.2	6.6

Note: CD3-counts based on single cells in the gate

Table 4: Thymus Analysis

	% donor type (dt)	% dt+ CD45+	% dt+ CD3+
Mouse 2	0.8	1.1	1.1
Mouse 3	0.6	1.2	1.7
Mouse 5	1.3	1.4	1.8
Mouse 6	0.4	0.5	0.7
Mouse 7	0.4	0.8	1
Mouse 8	0.4	0.8	1.2
Mouse 9	1	1.7	2.1
Mouse 10	1	1.2	1.6
Mouse 11	0.8	0.8	1.5
Mouse 12	0.4	0.5	0.8
Mouse 14	0.5	0.6	1.5
Mouse 18	0.5	0.5	0.8

Table 5: Peripheral Blood Analysis

	% donor type (dt)	% dt+ CD45+	% dt+ B220+	% dt+ GR1+	% dt+ CD11b+	% dt+ CD3+
mouse 2	16.7	20.6	1.4	4.5	89.2	1
mouse 3	17.3	15.5	1.1	2.7	80	4.9
mouse 5	17.6	17.3	0.8	5	87.3	0.8
mouse 6	8.9	8.5	0.7	2		0.5
mouse 7	17.9	17.5	1.3	4.5		0.7
mouse 8	10.8	10.5	0.9	4	80.9	2.3
mouse 9	12.9	13.3	0.9	2.7		1.1
mouse 10	5	3.9	0.8	3.3		4.3
mouse 11	9	8.8	0.8	4.2		0.8
mouse 12	35.8	35.6	1.1	1.9	84.2	2.4
mouse 14	9.7	9.4	0.8	3		0.4
mouse 18	10.4	9.6	1.1	3.1	80.2	1.4

Abbrevations:

dt	cells of donor type CD45.2 (Ly 5.2)
CD45	pan-leukocyte marker for all white blood cells
c-kit	marker of very early (pluripotent, reconstituting) stem cells
B220	marker for B cells of all maturation stages
Ter119	marker for all erythrocytic (red blood) stages
CD3	marker for all T lymphocytes including CD4 and CD8 positive cells
GR1	marker for granulocytes
CD11b	commonly used as myeloid marker (white blood cells) but also detected on lymphoid cells

[0069] Example 6 RT-PCR for Cultured hMesSCs

[0070] hMesSCs were cultured as described above for differentiation into HSC and endothelial cells. Cells were harvested for immunoflorescence as described above and for RT-PCR.

[0071] For RT-PCR, RNA was extracted using the Invisorb Spin Cell-RNA® Mini-kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. RNA was stored at –80 °C. Reverse transcription (RT) of extracted RNA was performed using the bulk First-strand c-DNA synthesis kit (Amersham, Freiburg, Germany). The cDNA was stored at –20 °C. For the PCR reaction 5 µl cDNA-template was mixed with 2.5 µl 10 x PCR-buffer, 0.5 µl 10 mM dNTP´s, 0.5 µl of each primer (50 ng/µl), and 0.5 µl polymerase (Ampli-Taq., Gibco) in a total volume of 25 µl for each probe. PCR was carried out in a programmable Biometra Uno-Thermobloc (Biometra, Göttingen, Germany) using the primers shown in Table 6. Negative controls were performed for each set of primers including water instead of cDNA. Samples were analyzed on 2 % agarose gels. The size of the PCR-fragments was estimated using the DNA-standard marker VIII (Gibco). Primers were synthesized by MWG Biotech (Ebersberg, Germany) according to the sequences in the Table 6.

[0072] Results of the RT-PCT showed gene expression detected using primers for CD41B, Notch-1, GATA2, GATA3, Runx1, SCL, and CD117 (c-kit) that encode lineage restricted hematopoietic transcription factors. SCL, GATA-1 and GATA-2 are expressed in multipotent progenitors prior to lineage commitment, but are down-regulated during granulocyte/monocyte differentiation RT-PCR results for cells up to 2 passages also showed weak bands for CD14 and CD34 which were not detected in cells in later passages.

Table 6: RT-PCR Primers and Conditions

Marker	Product size	Primer	Sequence	SEQ ID NO
CD34	149 bp	F	CTG CTC CTT GCC CAG TCT G	1
		R	GAA TAG CTC TGG TGG CTT GCA	2
CD133	591 bp	F	CCA CGA CTG TCG TAGCAG GT	3
		R	GCT GTT CTG CAG GTG AAG AG	4
CD14	289 bp (619)	F 1,2	GCT GTT CTG CAG GTG AAG AG	5
		R 1	CCT CTA CTG CAGACA CAC A	6
	619 bp	R 2	GGC TTG AGC CAC TGC AGC	7
CD16	741 bp	F	CCT CAA TGG TAC AGC GTG C	8
		R	GGG TTG CAA ATC CAG AGA AA	9
CD16	480 bp	F	GGA CAA TTC CAC ACA GTG G	10
		R	GGA GTA CCA TCA CCA AGC A	11
CD41B	383 bp	F	GTC AGC TGG AGC GAC GTC A	12
		R	GGA GTC AAA GGA GAG GCT C	13
Glycophorin A	884 bp	F	CCT TCA TTC TGA ACA GGC AA	14
		R	CCT TGG CAT TTG GGT CAT T	15
VWF	438 bp	F 1	GAG TGA GCC TCT CCG TGT A	16
		R 1	GCT CTT CAG AAG CTG GCA C	17
	263 bp (664)	F 2 (3)	CCA GAT TTG CCA CTG TGA TG	18
		R2	CCA CTT CTG GGA GAT GCG CA	19
		R3	CCA CAC TGC TCA GCA CGA AG	20
NOTCH-1	177 bp	F	GCA CTG CGA GGT CAA CAC	21
		R	AGG CAC TTG GCA CCA TTC	22
GATA1	173 bp	F	CAG AAA CGC CGA GGG TGA	23
		R	TTA GAA GAG GTG GAA GTT GGA GTC A	24
GATA2	182 bp	F	ACA ACC ACC ACC TTA TGG CG	25
		R	GCA TGC ACT TTG ACA GCT CC	26
GATA3	573 bp	F	CCT GCA GTC CCT TTC GAC	27
		R	GCA ACT GGT GAA CGG TAA	28
GATA4	612 bp	F	CGA CTT CTC AGA AGG CAGA	29
		R	GGG AGA CGC ATA GCC TTG T	30
Runx-1	403 bp	F	GGT CAA CTC AGT TCC AGA G	31
		R	CTG GTG CAC AGG TAA AAG	32
Tal1 (SCL)	386 bp	F	GGA GCA AAC ACA GTT GGA T	33
		R	GGC ATC AGA GAC TGT GCT T	34
CD117 c-kit	486 bp	F	GGA GAT CTG TGA GAA TAG GCT	35
		R	CAT ACA TTT CAG CAG GTG CG	36
KDR	626 bp	F	TAC TTG TCC ATC GTC ATG GAT CCA G	37
		R	CTG TAA CAG ATG AGA TGC TCC AAG G	38
Hemoglobin alpha 1	195 bp	F	CCA CAG ACT CAG AGA GAA C	39
		R	CCT TAA CCT GGG CAG AGC	40
Hemoglobin alpha 2	212 bp	F	CTG GAG AGG ATG TTC CTG T	41
		R	GCT TGA AGT TGA CCG GGT	42
c-MPL	666 bp	F	CCT GTA TAT AAT CCT CAC CAA	43
		R	GCT CAT CTG CAG GCA CTG	44

EPOR	311 bp	F	GCT GTA TCA TGG ACC ACC T	45
		R	GCT TCC ATG GCT CAT CCT	46
CD31	654 bp	F	GGA GTT TCC AGA AAT CAT AA	47
		R	GGA ATG GCA ATT ATC TGC AA	48

[0073] Although the invention herein has been described in connection with a preferred embodiment thereof, it will be appreciated by those skilled in the art that additions, modifications, substitutions, and deletions not specifically described may be made without departing form the spirit and scope of the invention as defined in the appended claims. It is therefore intended that the foregoing detailed description be regarded as illustrative rather than limiting, and that it be understood that it is the following claims, including all equivalents, that are intended to define the spirit and scope of this invention.

CLAIMS

1. A method of producing blood products *in vitro* comprising culturing isolated non-SV40 transformed mesenchymal stem cells with growth factors for a time sufficient to produce at least one type of blood products.

2. The method of claim 1 wherein said at least one type of blood products comprises myeloid stem cells.

3. The method of claim 1 wherein said at least one type of blood products comprises endothelial cells.

4. The method of claim 1 wherein said at least one type of blood products comprises lymphoid stem cells.

5. The method of claim 1 wherein said at least one type of blood products comprises dendritic cells.

6. The method of claim 1 wherein said at least one type of blood products comprises erythroid cells.

7. The method of claim 1 wherein said at least one type of blood products comprises megakaryocytes.

8. A method of treatment of a patient in need of a blood product, said method comprising

culturing isolated non-SV40 transformed mesenchymal stem cells *in vitro* with growth factors for a time sufficient to produce at least one type of blood products;

isolating said blood product; and

delivering a therapeutic amount of at least one type of blood products produced to said patient.

9. The method of claim 8 wherein said at least one type of blood products comprises myeloid stem cells.

10. The method of claim 8 wherein said at least one type of blood products comprises endothelial cells.

11. The method of claim 8 wherein said at least one type of blood products comprises lymphoid stem cells.

12. The method of claim 8 wherein said at least one type of blood products comprises dendritic cells.

13. The method of claim 8 wherein said at least one type of blood products comprises erythroid cells.

14. The method of claim 8 wherein said at least one type of blood products comprises megakaryocytes.

15. A method of differentiating non-SV40 transformed mesenchymal cells comprising:

culturing isolated mesenchymal stem cells *in vitro* with growth factors; and

producing at least one type of blood cell products.

16. The method of claim 15 wherein said at least one type of blood products comprises myeloid stem cells.

17. The method of claim 15 wherein said at least one type of blood products comprises endothelial cells.

18. The method of claim 15 wherein said at least one type of blood products comprises lymphoid stem cells.

19. The method of claim 15 wherein said at least one type of blood products comprises dendritic cells.

20. The method of claim 15 wherein said at least one type of blood products comprises erythroid cells.

21. The method of claim 15 wherein said at least one type of blood products comprises megakaryocytes.

22. Use of at least one type of blood products obtainable by culturing isolated non-SV40 transformed mesenchymal stem cells *in vitro* with growth factors for a time sufficient to produce at least one type of blood products for preparing a pharmaceutical composition for treating leukemia, thrombocytopenia, leukopenia, granulocytopenia, lymphocytopenia, aplastic anemia, and/or autoimmune disease with or

without bone marrow involvement, for treating patients after chemotherapy, total body irradiation or irradiation of single parts of the body, for treating vascular, ischemic and/or malignant diseases, or anemia.

23. Use of at least one type of blood products obtainable by culturing isolated non-SV40 transformed mesenchymal stem cells *in vitro* with growth factors for a time sufficient to produce at least one type of blood products for preparing a pharmaceutical composition for diagnosis of cancers and metastasis, wherein said at least one type of blood products is labelled with radioactive compounds.

24. The use of claim 22 or 23, wherein said at least one type of blood products comprises myeloid stem cells.

25. The use of claim 22 or 23, wherein said at least one type of blood products comprises endothelial cells.

26. The use of claim 22 or 23, wherein said at least one type of blood products comprises lymphoid stem cells.

27. The use of claim 22 or 23, wherein said at least one type of blood products comprises dendritic cells.

28. The use of claim 22 or 23, wherein said at least one type of blood products comprises erythroid cells.

29. The use of claim 22 or 23, wherein said at least one type of blood products comprises megakaryocytes.

30. Blood product obtained according to the method of any of claims 1 to 7 or 15 to 21.

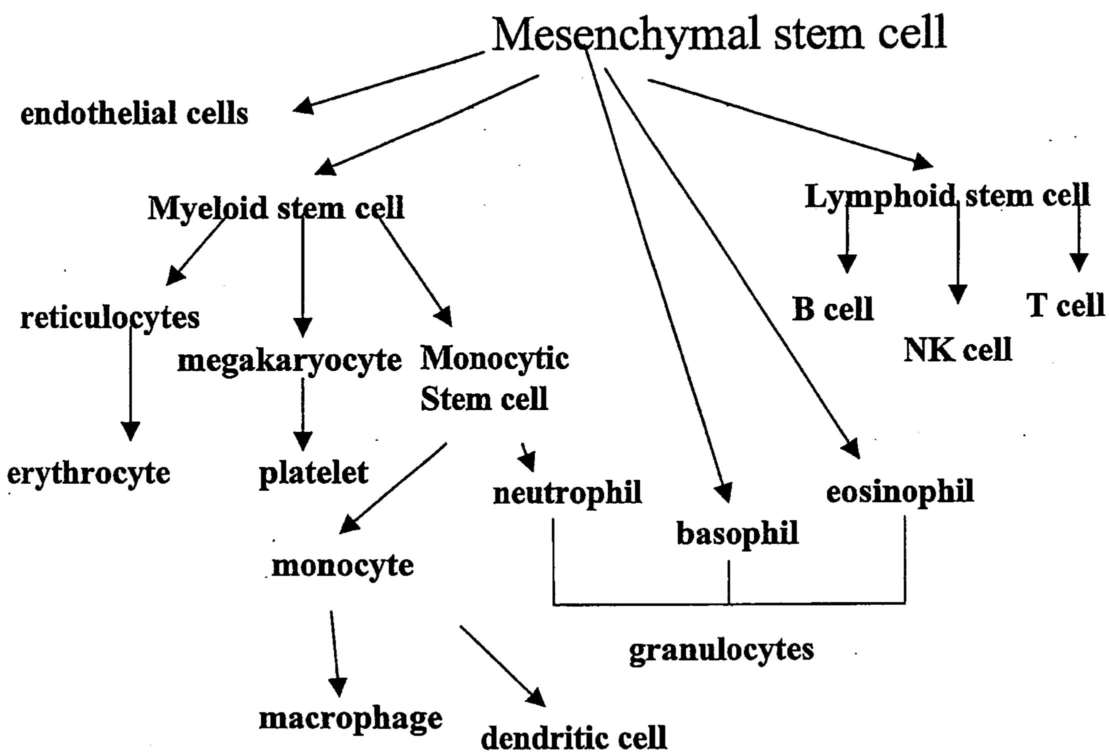
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Figure 1

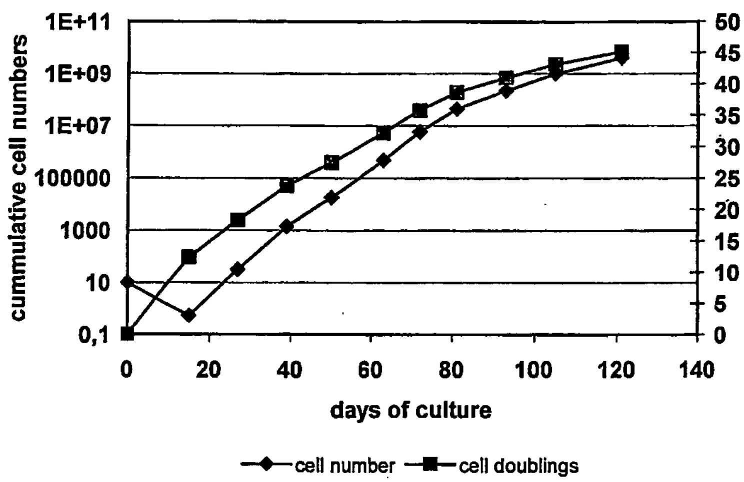

Figure 2: Growth and doubling of MSC in vitro

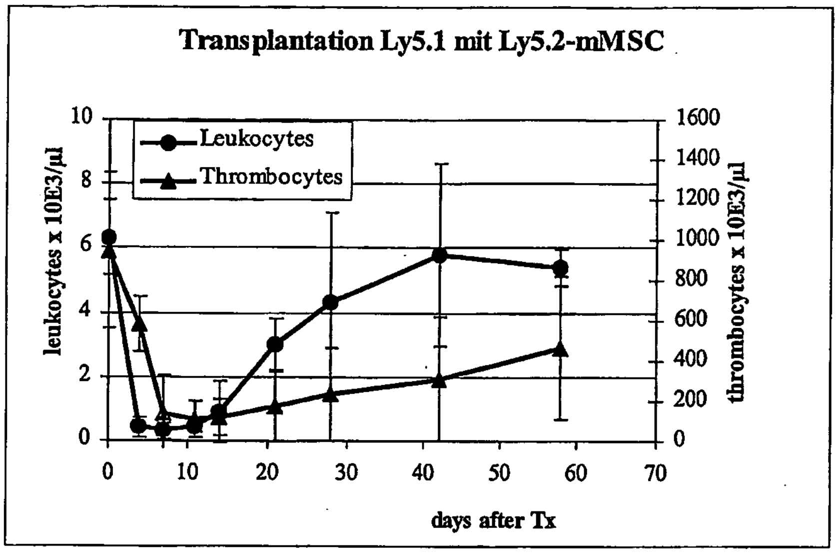

Figure 3: Blood cell counts after transplantation with mMesSCs

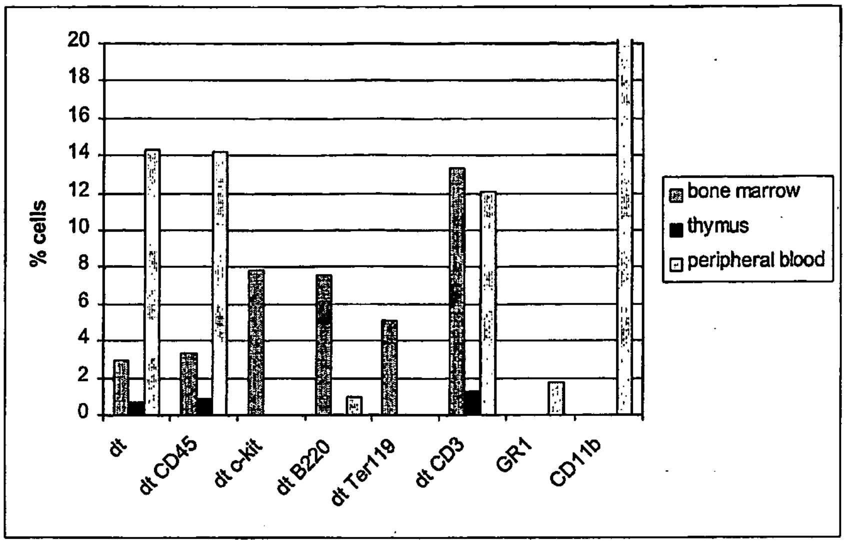

Figure 4: Analysis of subpopulation of transplanted mice
Abbreviations: dt, donor type.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/011570

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 A61K35/18 A61K35/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/61588 A (OSIRIS THERAPEUTICS INC) 2 December 1999 (1999-12-02) page 19, line 11 - line 31	1,2,7-9, 14, 22-24, 29,30
X	PEI XUETAO: "Stem cell engineering:The new generation of cellular therapeutics" INTERNATIONAL JOURNAL OF HEMATOLOGY SUPPL.I, vol. 76, August 2002 (2002-08), pages 155-156, XP009042425 page 156, column 1, paragraph 2	1,2,4-7, 15,16, 18-21,30

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 January 2005	02/02/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Renggli-Zulliger, N

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/011570

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OSAWA M ET AL: "Long-term lymphohematopoietic reconstitution by a single CD34 low/negative hematopoietic stem cell" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 273, no. 5272, 12 July 1996 (1996-07-12), pages 242-245, XP002097289 ISSN: 0036-8075 abstract; figure 3 -----	22
X	HU YING ET AL: "Transplantation of mesenchymal stem cells followed by G-CSF injection can reconstitute hematopoiesis of lethally irradiated BALB/C mice" BLOOD, vol. 98, no. 11 Part 2, 16 November 2001 (2001-11-16), page 316b, XP009042610 & 43RD ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY, PART 2; ORLANDO, FLORIDA, USA; DECEMBER 07-11, 2001 ISSN: 0006-4971 abstract -----	22
X	JIANG YUEHUA ET AL: "Pluripotency of mesenchymal stem cells derived from adult marrow" NATURE (LONDON), vol. 418, no. 6893, 4 July 2002 (2002-07-04), pages 41-49, XP001204372 ISSN: 0028-0836 abstract; figure 3 -----	1-22, 24-30
X	REYES MORAYMA ET AL: "Origin of endothelial progenitors in human post-natal bone marrow" BLOOD, vol. 98, no. 11 Part 1, 16 November 2001 (2001-11-16), page 821a, XP009042605 & 43RD ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY, PART 1; ORLANDO, FLORIDA, USA; DECEMBER 07-11, 2001 ISSN: 0006-4971 abstract -----	1-22, 24-30
A	WO 03/070922 A (HA CHUL-WON ; YANG SUNG-EUN (KR); YANG YOON-SUN (KR); MEDIPOST CO LTD) 28 August 2003 (2003-08-28) the whole document -----	1-30

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/011570

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PEI X: "Who is hematopoietic stem cell: CD34+ or CD34-?" INTERNATIONAL JOURNAL OF HEMATOLOGY. DEC 1999, vol. 70, no. 4, December 1999 (1999-12), pages 213-215, XP009042521 ISSN: 0925-5710 abstract -----	1-30

INTERNATIONAL SEARCH REPORT

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 8-14 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2004/011570

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9961588	A	02-12-1999	AU	4094099	A	13-12-1999
			CA	2328425	A1	02-12-1999
			EP	1078042	A1	28-02-2001
			WO	9961588	A1	02-12-1999
			US	6225119	B1	01-05-2001
			US	2001005591	A1	28-06-2001
WO 03070922	A	28-08-2003	EP	1483371	A1	08-12-2004
			WO	03070922	A1	28-08-2003
			KR	2003069115	A	25-08-2003